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N-Acetyl-L-cysteine suppresses TGF-β signaling at distinct molecular steps: The biochemical and biological efficacy of a multifunctional, antifibrotic drug

Steffen K. Meurer, Birgit Lahme, Lidia Tihaa, Ralf Weiskirchen *, Axel M. Gressner *

Institute of Clinical Chemistry and Pathobiochemistry, RWTH University Hospital Aachen, Pauwelsstr. 30, D-52074 Aachen, Germany

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Abstract

The interrelated signaling via TGF-β1 and reactive oxygen species has a profound impact on fibrogenesis and is therefore selected as target for antifibrotic therapies. This prompted us to investigate the influence of the antioxidant *N*-acetyl-L-cysteine on TGF-β signaling in culture-activated hepatic stellate cells, the most relevant pro-fibrogenic cell type in liver. Dissection of the molecular steps involved in TGF-β signaling revealed that *N*-acetyl-L-cysteine dose-dependently abrogated the induction of the TGF-β1 signaling reporter gene activation, the phosphorylation of Smad2 and Smad3, and the up-regulation of Smad7 mRNA. By means of Western blot analysis and cross-linking experiments, it was demonstrated that these effects are based on disintegration of TGF-β1 and the TGF-β receptor endoglin, as well as a reduced ligand binding capacity of betaglycan. We conclude that *N*-acetyl-L-cysteine is a specific inhibitor of TGF-β signaling targeting different components of the TGF-β signaling machinery. In conclusion, these findings suggest that this non-toxic aminothiol downregulates TGF-β signal transduction thereby mediating beneficial effects on experimental liver fibrosis characterized by TGF-β hyperactivity.
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Keywords: Fibrosis; Liver; Reactive oxygen; Receptor signaling; Smad

1. Introduction

In liver, the activation of hepatic stellate cells (HSC) results in transdifferentiation of this fibrogenic precursor cell type to the extracellular matrix-producing myofibroblastic phenotype (MFB). This process is regarded as a key issue in fibrogenesis and is therefore in the focus of therapeutic strategies [1]. Numerous studies have identified TGF-β as the major profibrogenic master cytokine, which in concert with other growth factors promotes transdifferentiation of HSC into MFB, stimulation of matrix gene expression, downregulation of matrix degradation and induction of hepatocellular apoptosis, thereby promoting the numerical expansion of MFB. Due to the pleiotropic fibrogenic effects of TGF-β, therapeutic strategies were developed to antagonize or block synthesis of the ligand [2–6], to interfere with the intracellular Smad signaling pathway [7], and to suppress generation and extracellular activation of TGF-β by MFB and other liver cell types [8].

Of particular interest is the interrelation of TGF-β signaling and reactive oxygen species (ROS) formation; in cultured HSC, TGF-β increases the production of H_2O_2 [9], which in turn induces the expression of α1(I) procollagen mRNA [10]. Catalase, an enzymatic scavenger of H_2O_2, abrogated TGF-β mediated type I collagen gene expression [10], supporting the hypothesis that H_2O_2 might act as a mediator of TGF-β signaling. Similarly, H_2O_2 was identified as mediator in acetaldehyde-induced α1(I) collagen gene expression [11]. Direct profibrogenic effects of H_2O_2 were also observed in co-cultures of HSC with HepG2 cells overexpressing CYP2E1 [12]. Furthermore, the interruption of the TGF-β autocrine loop by expression of soluble transforming growth factor beta type II receptor

Abbreviations: Ad-CA-ALK-5, adenovirus expressing a constitutively active TGF-β receptor type I; BSO, L-buthionine-*S*, *R*-sulfoximine; DTT, dithiothreitol; FCS, fetal calf serum; HSC, hepatic stellate cell(s); Luc, luciferase; MLP, major late promoter; MOI, multiplicity of infection; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; TGF-β, transforming growth factor β; TβR, TGF-β receptor

* Corresponding authors. Tel.: +49 241 8088678; fax: +49 241 8082512.
E-mail addresses: rweiskirchen@ukaachen.de (R. Weiskirchen), agressner@ukaachen.de (A.M. Gressner).

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was recently shown to inhibit oxidative stress in activated HSC [13].

N-Acetyl-L-cysteine (NAC), a non-toxic aminothiol and synthetic precursor of intracellular glutathione (GSH), is clinically used as a harmless, powerful antioxidant capable of increasing the defense mechanisms against ROS [14]. Pharmacological actions include repletion of intracellular GSH stores, scavenging of toxic radicals, suppression of tumour necrosis factor-α (TNF-α) production, and stimulation of cathepsin B-mediated cleavage of platelet-derived growth factor receptor type β [15]. In cultured HSC it was shown that NAC induces cell cycle arrest through its reducing activity and by redox-mediated extracellular proteolysis of platelet-derived growth factor receptor type β [15–17]. Moreover, in vivo the naturally occurring amino acid L-cysteine and its derivative NAC were recently suggested to diminish dimethylnitrosamine-induced liver fibrosis by inhibiting the activation and proliferation of HSC [18,19].

So far, the effect of NAC action on TGF-β induced fibrogenic responses in HSC cultures has not been determined at the molecular level. In this study, we addressed experiments to this question. The data point to NAC elicited disaggregation of the TGF-β1 dimer, a reduced TGF-β1 binding activity of the transforming growth factor type III receptor (TβRIII) betaglycan, and a decomposition of a second accessory TβRIII, i.e. endoglin, recently shown to be expressed in HSC [20]. Furthermore, the demonstration that these alterations are not prevented by administration of proteinases and proteasome inhibitors suggest that the disintegration of individual signaling components is not proteolytically induced. Therefore, the presented data indicate for the first time, that (i) the protective role of the antioxidant agent NAC against the development of hepatic fibrosis is based on a direct blockade of TGF-β1 function and signaling, and (ii) that these effects are direct contributable to the chemical characteristics of NAC.

2. Materials and methods

2.1. Isolation and culture of hepatic stellate cells

HSC were isolated from male Sprague–Dawley rats and cultured as previously described [21].

2.2. Adenoviral infection of HSC and reporter assays

Adenoviral stocks were prepared following standard protocols outlined in detail elsewhere [6]. In brief, adenoviruses were purified by a two-step-procedure. Firstly, the viral particles were concentrated by CsCl density gradient centrifugation. Secondly, the viruses were further purified through the BD Adeno-X™ Purification Filter system (BD Biosciences, Clontech). The infections were performed at an MOI of 50, and after infection, cells were serum-starved at 0.5% FCS for 16 h, preincubated with or without NAC (Fluimucil Antidot; Zambon) followed by TGF-β-stimulation as indicated. Luciferase (Luc) activity was measured with the Steady-Glo Luciferase Assay (Promega), according to the manufacturer's protocol. The constitutively active TGF-β receptor I (Ad-CA-ALK-5) was kindly provided by Dr. A. Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Details of adenoviral constructs used in this study are given in Supplementary Fig. S1.

2.3. Isolation of total RNA and real time RT-PCR

HSC at the third or fourth day of primary culture were treated with NAC and TGF-β1 for indicated time periods. Total RNA was purified using the RNeasy Mini Kit (Qiagen) and quantitative analysis of Smad7 mRNA was performed as previously described [22].

2.4. Transient transfections

COS-7 cells were plated into 6-well dishes and transfected with 2 μg of the expression plasmids pcDNA-endoglin [20] or pCMV-HA-TβRIII [23] using the FUGENE 6™ method (Roche). After 24 h, the medium was renewed, and cells were extracted one day later in lysis buffer [50 mmol/l Tris/HCl (pH 7.2), 250 mmol/l NaCl, 2% (v/v) Nonidet P-40, 0.1% (w/v) sodium dodecylsulfate (SDS), 0.5% (w/v) sodium deoxycholate, 2.5 mM EDTA] for SDS-PAGE.

2.5. Affinity labeling, cross-linking of TGF-β receptors, and TGF-β1 cleavage assay

Affinity labeling with [^{125}I]-TGF-β1 (Amersham Pharmacia Biotech.) and cross-linking experiments were performed as described previously [20]. [^{125}I]-TGF-β1 was incubated with indicated concentrations of NAC or dithiothreitol (DTT) and reaction products were separated on non-reducing SDS-PAGE followed by autoradiography.

2.6. Western blotting

Protein extracts were separated on 4-12% bis–Tris gels (Invitrogen) using MOPS [50 mmol/l 3-(*N*-morpholino) propane sulfonic acid (MOPS), 50 mmol/l Tris–HCl, 3.47 mmol/l SDS, 1.025 mmol/l EDTA; pH 7.7], or MES-running buffer [50 mmol/l 2-(*N*-morpholino) ethane sulfonic acid (MES), 50 mmol/l Tris–HCl, 3.47 mmol/l SDS, 1.025 mmol/l EDTA; pH 7.3], electroblotted on nitrocellulose membranes (Schleicher & Schuell) and treated with polyclonal antibodies against phospho-Smad2 (generous gifts from Dr. Peter ten Dijke, The Netherlands Cancer Institute, Amsterdam, The Netherlands), TβRI [sc-398], TβRII [sc-400], or TβRIII [sc-6199], RAGE [sc-8230] (all from Santa Cruz Biotechnology Inc.), or an endoglin-specific antibody prepared against peptide LALHPSTLSQEVY coupled to the keyhole limpet hemocyanin. The antibodies were all diluted in 2.5% (w/v) non-fat milk powder in TBST

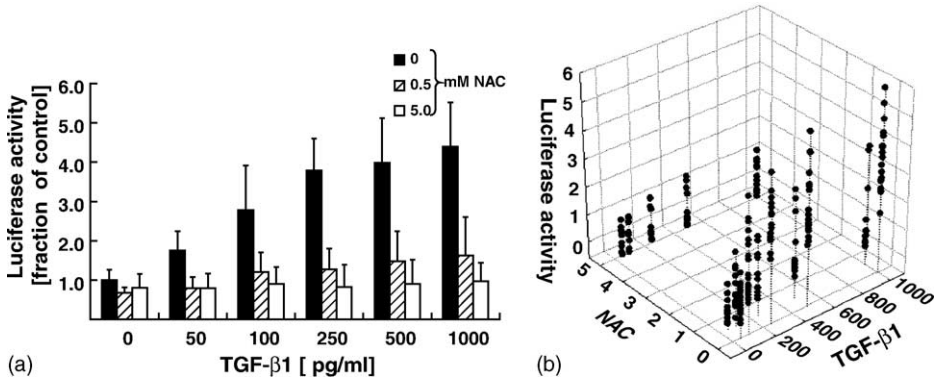

Fig. 1. NAC dose-dependently inhibits TGF-β1-induced reporter gene activity. (a) HSC were infected with 50 multiplicity of infection (MOI) Ad-$(CAGA)_9$-MLP-Luc and incubated with 0.5 or 5 mM NAC for 1.5 h prior exposition with indicated concentrations of TGF-β1 for 3 h. Control incubations received no NAC. Mean values ± S.D. of luciferase activities (given as fraction of control) of four independent experiments are shown. (b) The statistical relevance was demonstrated in multiple linear regression analysis ($p < 0.001$). Regression coefficients are: $r = 0.326$ (TGF-β effect) and $r = 0.54$ (NAC effect).

buffer [10 mM Tris–HCl, pH 7.6; 150 mM NaCl; 0.1% (w/v)] Tween 20] and blots were incubated with secondary antibodies (Santa Cruz), conjugated with horseradish peroxidase (HRP) or alkaline phosphatase (AP) and developed using the Supersignal™ (Perbio Science) for HRP and the Western-Star™ protein detection kit (TROPIX) for AP.

2.7. Identification of free sulfhydryl groups in NAC-treated TβRIII

HSC grown with or without NAC for 3 h were solubilized in lysis buffer [50 mmol/l Tris/HCl (pH 8.5), 250 mmol/l NaCl, 2% (v/v) Nonidet P-40, 0.1% (w/v) sodium dodecylsulfate (SDS), 0.5% (w/v) sodium deoxycholate, 2.5 mM EDTA] and incubated with EZ-Link™ PEO-iodoacetyl biotin beads (Perbio Science), solubilized in reaction buffer [25 mg/ml in 50 mmol/l Tris–HCl pH 8.5, 5 mmol/l EDTA] for 16 h at 4 °C and then incubated with 50% (v/v) slurry in reaction buffer UltraLink™ Immobilized NeutrAvidin™ (Perbio Science). After centrifugation, beads were washed with lysis buffer and PBS, respectively. The pellet was resuspended in LDS sample buffer and heated at 70 °C for 10 min. After centrifugation, proteins containing sulfhydryl groups were subjected to SDS-PAGE and Western blot analysis to detect TβRIII.

2.8. Statistical analysis

The statistical analysis was performed using the Statistica software version 6.0 (StatSoft, Tulsa, OK). Details of each analysis are indicated in the respective figure legends.

3. Results

3.1. NAC inhibits TGF-β-dependent reporter gene expression

HSC were adenovirally infected with a highly sensitive TGF-β reporter construct, i.e. Ad$(CAGA)_9$-MLP-Luc, comprising artificial Smad3/Smad4 binding sites (CAGA-box) derived from the plasminogen activator inhibitor-1 (PAI-1) promoter and part of the major late promoter [24]. Infected HSC were treated with 0.05–1 ng/ml TGF-β1 or left untreated in the absence or presence of 0.5 or 5 mM NAC as indicated (Fig. 1a). The luciferase activity was determined 3 h thereafter showing a dose-dependent stimulation of measured reporter activity. This stimulation was significant decreased (0.5 mM NAC) or abolished (5 mM NAC) by the supplementation with NAC prior to the addition of TGF-β1 (Fig. 1b) revealing that NAC suppresses TGF-β signaling.

3.2. NAC inhibits TGF-β-induced Smad2 and Smad3 phosphorylation

We next addressed the question, if TGF-β-responsiveness of HSC as measured by phosphorylation of Smad2 (pSmad2) and Smad3 (pSmad3) can be omitted by NAC. Therefore, cells were pretreated with 5 mM NAC for 90 min, incubated with TGF-β1 (1 ng/ml) for 1 h, and protein extracts were analyzed for TGF-β1 induced Smad2 and Smad 3 phosphorylation (Fig. 2). The data strengthen the conclusion that NAC interferes with TGF-β signaling by preventing the phosphorylation of the receptor Smads.

3.3. NAC inhibits the expression of antagonistic Smad7 and receptor for advanced glycation end products (RAGE)

To complement the inhibitory effect of NAC on TGF-β mediated downstream effects, we examined its influence on Smad7 expression, which is rapidly and transiently inducible by TGF-β1 in HSC and other cell types [25]. In the experimental setting, HSC were pretreated with 5 mM NAC for 1.5 h and then exposed to TGF-β1 (1 ng/ml) for 1 h. NAC was found to abolish completely the 1.5 fold stimulating effect of TGF-β1 on Smad7 mRNA expression (Supplementary Fig. S2a). Moreover, the

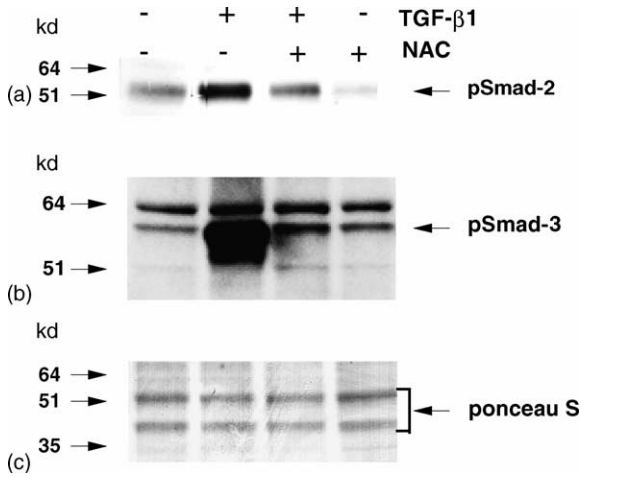

Fig. 2. NAC inhibits TGF-β1-dependent phosphorylation of Smad2 and Smad3 in primary cultured HSC. Phosphorylation of Smad2 (pSmad2) (a) and Smad3 (pSmad3) (b) were detected by immunoblotting of whole cell lysates. Extracts were prepared after pretreatment of HSC with or without NAC, respectively, for 90 min followed by incubation with or without 1.0 ng/ml TGF-β1 for 1 h. (c) Staining with ponceaus S were used as internal loading controls.

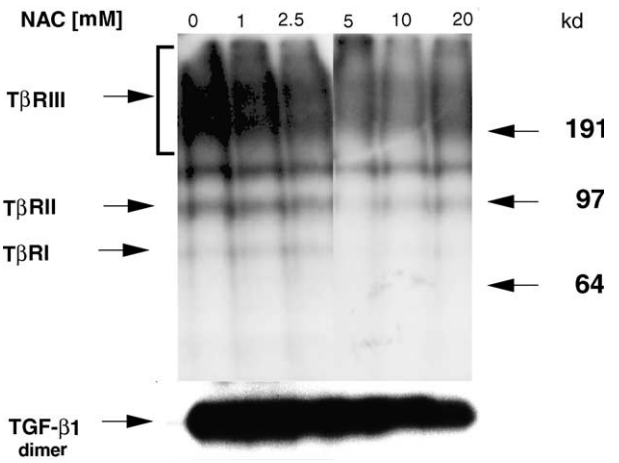

Fig. 3. NAC effects on TGF-β1 binding to its signaling receptors. HSC cultured for four days were exposed to [^{125}I]-TGF-β1 after treatment with indicated concentrations of NAC for 2 h, chemically cross-linked, separated using SDS-PAGE, and visualized by autoradiography. Arrows indicate the positions of receptor ligand complexes. Molecular weights (kd) are indicated on the right. Control experiments that include a 200-fold excess of unlabeled TGF-β1 do not show any labeling with [^{125}I]-TGF-β1 (not shown).

induction of the de novo synthesis of the RAGE protein, previously shown in our laboratory to be time-dependently induced by TGF-β1 [26], is suppressed in the presence of NAC as analyzed by means of Western blot analysis (Supplementary Fig. S2b).

3.4. NAC acts at the receptor level, independent of proteases

As shown in Fig. 2 even the first step in intracellular TGF-β-signaling, i.e. the phosphorylation of receptor Smads by TβRI, is abolished by NAC. To elucidate if the activation of TGF-β receptors is hampered by NAC we co-infected HSC with Ad(CAGA)$_9$-MLP-Luc and an adenovirus expressing a constitutively activated TGF-β receptor type I (Ad CA-ALK-5) [27]. The resulting luciferase activity was measured in dependence of NAC-treatment showing no inhibitory effect of NAC up to a concentration of 5 mM suggesting that NAC does not influence signaling of the constitutive active TGF-β receptor type I and influences processes in which TGF-β is directly involved in (Supplementary Fig. S3). Contrarily, the stimulation by exogenous ligand (TGF-β1) was dose-dependently inhibited suggesting that NAC interferes directly with TβR function. In case of PDGF-signaling it has been shown that NAC is able to trigger the proteolytical cleavage of PDGF-receptor β [15]. So we next asked whether proteinases might be involved in the mechanism of NAC action on the TGF-β-receptors. The data displayed that the broad-spectrum proteinase inhibitor leupeptin could neither abolish nor reduce the inhibitory effect of NAC on TGF-β-induced reporter gene expression (Supplementary Fig. S4).

3.5. NAC induced a decrease in TβRIII ligand binding and a decline in available bioactive ligand

To specify the receptor species which is affected by NAC we performed affinity labeling of cell surface TGF-β receptors with [^{125}I]-TGF-β1 and subsequent chemical cross-linking of HSC preincubated with NAC for 2 h. After that period NAC dose-dependently reduced the ligand binding to a high molecular weight receptor complex (Fig. 3), most likely comprising two type III receptors, betaglycan and endoglin [20]. Interestingly, when we analyzed the supernatant of cross-linking experiments, we found that in the presence of NAC, the disulfide-linked, dimeric ligand TGF-β1 itself was dose-dependently disintegrated into the biologically inactive monomer (Fig. 4a). In line with our findings, White et al. [28] postulated that the biological activity of TGF-β was significantly reduced when cells were cultured in the presence of 10 mM NAC for 24 h [28]. We here undoubtedly demonstrated the decomposition of TGF-β1 in vitro by incubating [^{125}I]-TGF-β1 with NAC or other chemicals with reducing attributes, e.g. dithiothreitol (DTT) (Fig. 4b), revealing that this difference in electrophoretic mobility is induced by breakage of the disulfide bonds.

3.6. NAC gives rise to a persistent decrease in ligand affinity of the type III receptor (TβRIII) complex

To figure out if the inactivation of the ligand is primarily responsible for the reduced labeling of the TβRIII-complex, we performed cross-linking experiments in which we preincubated cells for 2 h with 15 mM NAC while presenting TGF-β1 in the absence of NAC (Fig. 5). This

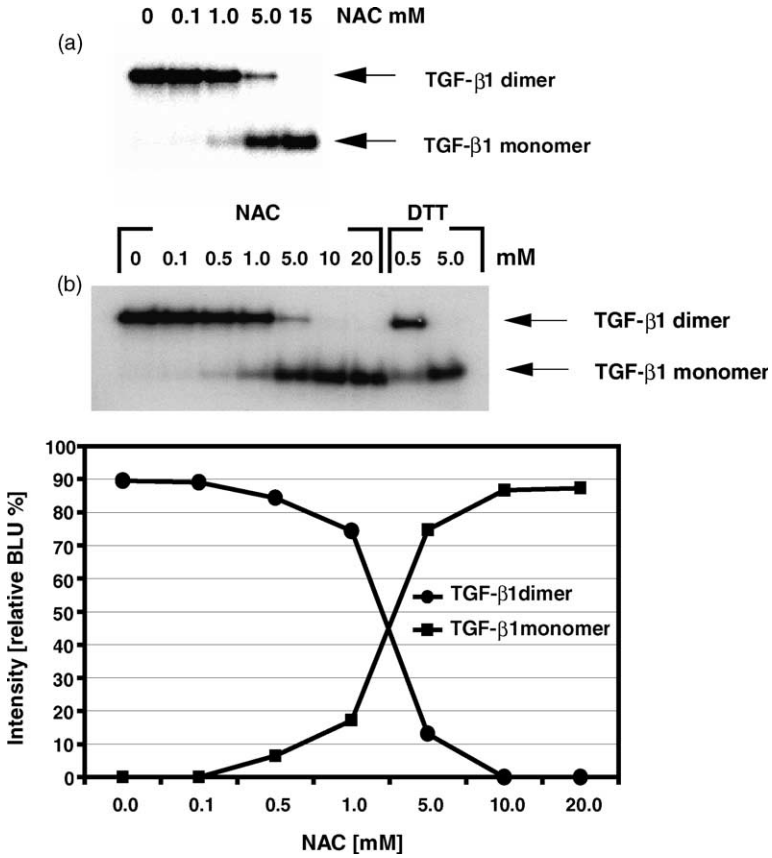

Fig. 4. NAC effect on TGF-β1. Supernatants taken from cross-linking experiments (a) or [^{125}I]-TGF-β1 incubated for 10 min at room temperature with indicated concentrations of NAC or DTT (b) were separated on SDS-PAGE and analyzed by autoradiography.

experiment revealed that although the offered integrity of the ligand in the assay is preserved (Fig. 5a) and that the labeling of the TβRIII-complex is still reduced (Fig. 5b). To substantiate this finding, we repeated the reporter assay (cf. Fig. 1) but applied the ligand in the absence of NAC (Supplementary Fig. S5). At least a preincubation with 2.5 mM NAC leads to a significant reduction in luciferase activity, confirming that NAC most likely induces a structural modification of type III receptors causing a decrease in ligand binding capacity. Furthermore, subsequent studies reveal that beside the labeling of the receptor and the activation of the artificial reporter construct also phosphorylation of Smad2 is reduced after administration of TGF-β prior to NAC treatment (not shown).

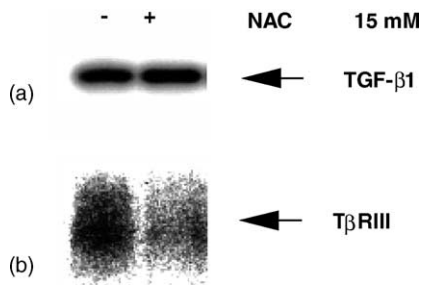

Fig. 5. NAC effect on TβRIII function. HSC cultured for four days were preincubated with NAC for 2 h, washed, exposed to [^{125}I]-TGF-β1, chemically cross-linked, separated by SDS-PAGE, and visualized by autoradiography. The integrity of the ligand present in the supernatant after the binding is dipicted in (a) and the TβRIII-ligand complexes after cross-linking are shown in (b), respectively. Note that although the biologically active ligand is present, the binding to TβRIII is still reduced.

3.7. NAC leads to an alteration of the structure of TGF-β type III receptors

HSC express both types of TGF-β type III receptors, i.e. betaglycan and endoglin [22,20]. Analysis of endoglin derived from cultured HSC which have been treated with NAC (Fig. 6a) or from lysates of untreated cells supplemented with different concentrations of NAC or DTT before the separation in SDS-PAGE (Fig. 6b) revealed a difference in electrophoretic mobility which occurs at NAC concentration less than 5 mM (Fig. 6a and b). We assumed that NAC as well as DTT lead to a destruction of the disulfide-bridges destroying the dimeric structure of endoglin. This assumption was further verified by labeling the free sulhydryl groups of endoglin with a thiol reactive compound after NAC treatment (Supplementary Fig. S6). NAC led to a dose-dependent disappearance of the endoglin dimer (Supplementary Fig. S6a) accompanied by an

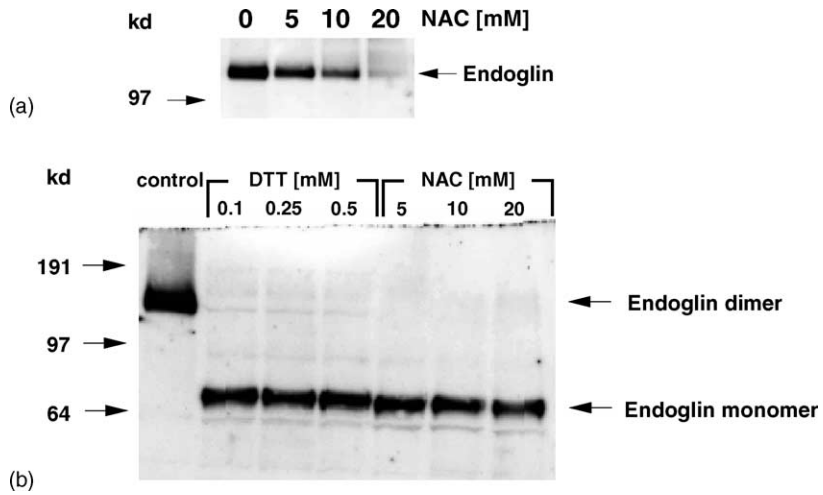

Fig. 6. NAC effect on the integrity of the TGF-β receptor type III (endoglin). Proteins of HSC cultured for four days were either preincubated with indicated concentrations of NAC for 1 h (a) or left untreated (b). Proteins were extracted in lysis buffer. Protein samples of cells preincubated with NAC were directly sunjected to SDS-PAGE (a). Protein lysates of untreated cells (b) were incubated for 5 min at room temperature with indicated NAC or DTT concentrations and separated by SDS-PAGE. Endoglin was detected by the antibody sc-6199.

increase of free available sulfhydryl-groups in the dimer (Fig. S6b) and monomer (Fig. S6c), respectively. In contrast to this Western blot analysis, the glycosylated form of betaglycan transiently expressed in COS-7 cells was not altered after NAC treatment (Supplementary Fig. S7, betaglycan). However, the unmodified core receptor revealed a slight shift to a higher molecular weight with increasing NAC concentrations (Supplementary Fig. S7, betaglycan core). This finding is most likely indicative for the loss of intramolecular cystins leading to a more open conformation with reduced ligand binding capacity as could be shown for the heterologously expressed betaglycan (Fig. 7).

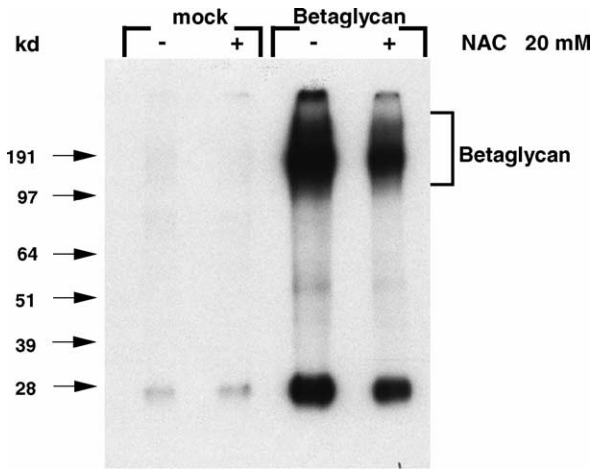

Fig. 7. NAC effect on functionality of transient expressed TβRIII (betaglycan). COS-7 cells were transiently transfected with the expression plasmid pCMV-HA-TβRIII (betaglycan) or left untransfected (Mock). Prior to the binding of [^{125}I]-TGF-β1, cells were incubated for 2 h with or without NAC. The bound ligand was chemically cross-linked to its receptor, separated by SDS-PAGE, and visualized by autoradiography.

4. Discussion

NAC is available as an over the counter supplement in health stores and in oral solutions that can be ingested or aerosolized and inhaled. As a non-toxic aminothiol it is suitable as a mucolytic agent [29], in the treatment of acetaminophen (paracetamol) poisoning [30], for reducing the incidence of contrast-induced nephropathy [31], to diminish the symptoms and duration of the flu and the common cold, and in the course of immunodeficiency virus (HIV) infection [32]. Furthermore, it is taken by bodybuilders causing temporary lower levels of glutathione, and was recently shown to reduce body fat without compromising glucose tolerance [33]. In addition, NAC application is opportune in the treatment of cocaine addiction and in the removal of mercury from the body [34,35]. Although this antioxidant is widely exerted, there are only few reports available analyzing the molecular events establishing these therapeutic attributes.

In this study, we show that NAC treatment of HSC dose-dependently suppresses TGF-β-induced $(CAGA)_9$-driven luciferase gene expression, TGF-β mediated transcriptional up-regulation of Smad7, and phosphorylation of the TGF-β mediators Smad2 and Smad3. The functional effects are associated with a specific alteration of the accessory TβRIII endoglin, a reduced ligand affinity of betaglycan, and a direct disintegration of TGF-β1. Furthermore, signaling by a constitutive active TβRI was not inhibited by NAC. Thus, present results suggest a distinct effect of NAC, even in cell lysates incubated for a short-time period with this cysteine derivative. Noteworthy, this alteration can also be induced by other antioxidants, e.g. DTT. Therefore, the disintegration is most likely due to the direct breakage of disulfide bonds rather than proteolytic cleavage events. In line with this hypothesis is the finding

that NAC influences the redox status of cysteine-residues in endoglin.

This molecular ''splitting'' observed in case of endoglin is mechanistically different from the proteolytic cleavage (shedding) of membrane bound receptors like betaglycan [36]. Proteolytic processing, leading to liberation of the extracellular ligand binding domain has been shown as regulator of TGF-β activity, because soluble betaglycan retained high affinity towards TGF-β, but did not enhance binding to membrane receptors [37]. The potent inhibitory function of betaglycan on TGF-β signaling has already been exploited in experimental therapeutic trials [38,39]. A previous study, investigating ectodomain cleavage and release (shedding) of TβRIII in rat lung membranes proposes an alternative mechanism [40]. This group found, that betaglycan is subject to reducing agents. They present evidence for a receptor core protein, which is susceptible to reducing influences due to two fragments held together by disulfide bond(s). The authors propose a model of the TβRIII domain structure in rat lung membranes, which contains two cleavage sites sensitive to shedding proteinases. The fragments of one of these cleavage sites are held together by a disulfide bond, rendering the structure sensitive to reducing agents. Thus, complete cleavage of the ectodomain in its central portion requires both proteinases and reducing influences. However, in our experiments, we never detected a fragmentation of betaglycan, irrespective if we analyzed the endogenous or heterologously expressed receptor. Therefore, we can exclude a shedding mechanism to be responsible for the reduced ligand binding to betaglycan. We favour a NAC mediated change in the tertiary structure of the receptor by resolution of cysteine bridges. Although this modification is manifested only in a slight difference in electrophoretic mobility, it is sufficient to lower the ligand affinity of the receptor significantly.

The finding that both, NAC and DTT, as similarly acting sulfhydryl reagents decompose endoglin underscores that the reducing properties of these chemicals are causative for the observed downregulation of TGF-β signaling in cultured HSC. It is well established that TGF-β signaling is contingent upon the concentration of individual TβRs [41]. Therefore, the deprivation or biological inactivation of one receptor is sufficient to induce a strong decay of the TGF-β induced signaling. Furthermore, it is evident that members of the TGF-β signaling pathway are subject to regulation by the ubiquitin/proteasome pathway [42]. Therefore, we tested the effect of well-known proteasome inhibitors (MG132 and lactacystin) on NAC triggered endoglin cleavage and found no influence (data not shown). Thus, NAC does not use a proteasome-dependent mechanism to exert its destructive effect on endoglin. A previous investigation has reported a mechanism, by which NAC blocks PDGF induced signaling pathways in HSC [15]. In this study, it was shown that NAC induced extracellular proteolysis of PDGF type β receptor, resulting in decreased sensitivity of HSC to PDGF-BB. The activation of the thiol-proteinase cathepsin B by NAC, independently of its H_2O_2 scavenging function, was suggested as a mechanism. However, this effect was dependent on high concentrations of NAC (20 mM) and required a long incubation time (24 h) [15]. These conditions are different from our experimental design, using lower doses and short-time incubations. We have presently no indication that the NAC-induced fragmentation of endoglin is mediated by proteinases. As a proof for the resolution of disulfide bonds as the cause for the NAC induced difference in the molecular weight of endoglin, we could show that NAC increases the number of free sulfhydryl groups in the endoglin core protein. Since rat endoglin contains an unusual high number of 16 cysteines [20], one might speculate that a change of the redox status of cysteine residues could decrease stability of endoglin structure. Presently, there is no detailed information available on the co- or post-translational processing of endoglin or betaglycan, which might involve limited proteolysis at specific cleavage sites resulting in fragments that are held together by disulfide bonds.

A very critical aspect which to keep in mind is the direct effect of reducing substances on the ligand TGF-β itself. The bioactive ligand is composed of two identical monomers connected by a single disulfide bond [43]. The loss of biological activity due to incubation of TGF-β with glutathione and NAC has been reported and it was shown by mass spectroscopy that the dimeric form of the ligand disappears after incubation with thiols [28]. Here, we show that the disappearance of the dimer coincides with the generation of the monomeric biologically inactive form. Therefore, experiments in which a disulfide-linked dimeric ligand (e.g. PDGF) is used under reducing conditions obtained results have to be evaluated carefully.

We focused on the type III receptors, because in our experimental setting they displayed an obvious affection by reducing agents. Whether NAC also disturbs the binding and activation of the type II and type I receptor could not be excluded. The detection of the latter two receptors in Western blots revealed that at least the primary structure is not affected by NAC (data not shown). Nevertheless, it has been reported that DTT is able to block the activation of the type I receptor, most likely by a modification of the conformation resulting in reduced ligand binding [44]. This receptor is not competent to induce phosphorylation of Smad3 in vitro. To answer the question if a similar effect is elicited by NAC, experiments are currently in progress.

NAC effects on TGF-β-treated HSC, the most important fibrogenic precursor cell type in liver, can be very advantageous for therapeutic purposes. In combination with the reported inhibition of PDGF-triggered HSC proliferation by NAC, the loss of TGF-β effects on target genes suggests this compound both as a powerful and harmless antifibrotic drug. Indeed, recent experimental studies have reported promising results in a model of dimethylnitrosamine-induced rat liver fibrosis [18]. Our data add evidence that NAC can blunt

some major functions of the profibrogenic master cytokine TGF-β on HSC activation. In this context, it is noteworthy that L-cysteine was recently shown to have antifibrotic effect on the activation process in cultured HSC and in an experimental model of liver fibrosis induced by administration of dimethylnitrosamine in rats [19]. Thus, the administration of a simple amino acid (L-cysteine) or their derivatives can be a potential approach to block TGF-β activity in the course of hepatic fibrosis. In this regard the experience in therapeutically usage of NAC in a number of disorders, the unknown toxicity at doses commonly used, and the intracellular deacetylation to the harmless amino acid cysteine are further argues for testing NAC as a specific antagonist for TGF-β1 function when this cytokine or its signal cascade is the causative agent triggering the disease process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2005.07.001.

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